



Topical treatment with anti-oxidants and Au nanoparticles promote healing of diabetic wound through receptor for advance glycation end-products

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ABSTRACT

Impairment in diabetic wound healing constitutes an enormous biomedical burden. The receptor for advanced glycation end-products (RAGE) expression in the diabetic cutaneous wound may play a key role. However, the relationship between RAGE expression and topical application of anti-oxidant agents with gold nanoparticles (AuNP) in cutaneous diabetic wounds remains unclear. We tested the 3–5 nm AuNP, epigallocatechin gallate (EGCG), and α -lipoic acid (ALA) could change the RAGE expression and be helpful in diabetic wound. The mixture of AuNP+EGCG+ALA (AuEA) significantly attenuated the AGE-induced RAGE protein expression in fibroblasts (Hs68). Topical EGCG+ALA (EA) and AuEA application accelerated wound healing on diabetic mouse skin and decreased the RAGE expression. Vascular endothelial growth factor but not angiopoietin-1 significantly increased after EA or AuEA treatment for 7 days. Angiopoietin-2 significantly decreased at day 7 in AuEA group. Furthermore, immunoblotting of diabetic wound tissue showed significant decrease of CD68 expression from day 3 to day 7. The results suggest that combination of AuNP, EGCG, and ALA significantly accelerated diabetic cutaneous wound healing through angiogenesis regulation and anti-inflammatory effects. Blockade of RAGE by anti-oxidant agents and nanoparticles may restore effective wound healing in diabetic ulcer.

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1. Introduction

Diabetes will increase free radicals *in vivo* and reduce anti-oxidation capacity; diagnosed cases of diabetes have gradually increased year by year and research on diabetes has attracted greater attention from the medical profession. Moreover, diabetes may also complicate a number of other medical situations. Diabetic ulcers are one of the major complications and the major site of ulcerations is found on the feet. Diabetic ulcers are a very serious medical problem because of the persistent pain involved and the chance of bacterial infection. AGEs (Advanced glycation end products) and RAGE (receptor for AGEs) result in oxidative stress and cause abnormal angiogenesis in wound healing meaning that there are no good treatment methods determined so far (Huijberts et al., 2008; Lee and Lee, 2007). Moreover, patients with severe ulcers coupled with infection must often times undergo amputation thereby further complicating a patient's quality of life.

Wound healing is usually a natural protection mechanism in human body and it can be divided into three phases: inflammation,

proliferation and reshaping. Its main characteristic is the gradual repair of lost extracellular matrix, which forms a part of the dermal layer of the skin (Gurtner et al., 2008; Lamers et al., 2011). However, metabolic disorders in diabetes hinder the normal steps of the wound healing process. Many histological studies have shown that diabetes can lead to long-term inflammation which then causes delayed maturation of granulation tissues and reduced wound parallel tension (Gurtner et al., 2008). The increased inflammation in the wound also inhibits angiogenesis making the wound unable to move into the proliferation phase. In addition to an extended wound healing time, various angiogenic factors around the tissues also perform abnormally because of under the influence of diabetes causing an incomplete development of new blood vessels. Impairment of wound healing causes a significant degree of global morbidity and mortality (Gurtner et al., 2008). The underlying pathophysiology of diabetic ulcer is multifactorial (Singer and Clark, 1999) and the cellular signaling mechanisms that regulate wound healing are poorly understood.

Recent findings suggested the key roles of certain anti-oxidant reagents, such as epigallocatechin gallate (EGCG) (Kim et al., 2008), in wound healing. EGCG is a polyphenol that exists abundantly in unfermented teas. Several epidemiological studies linked the consumption of tea with a decrease in cardiovascular diseases (Hertog et al., 1993). Alpha-lipoic acid (ALA) is also known as a

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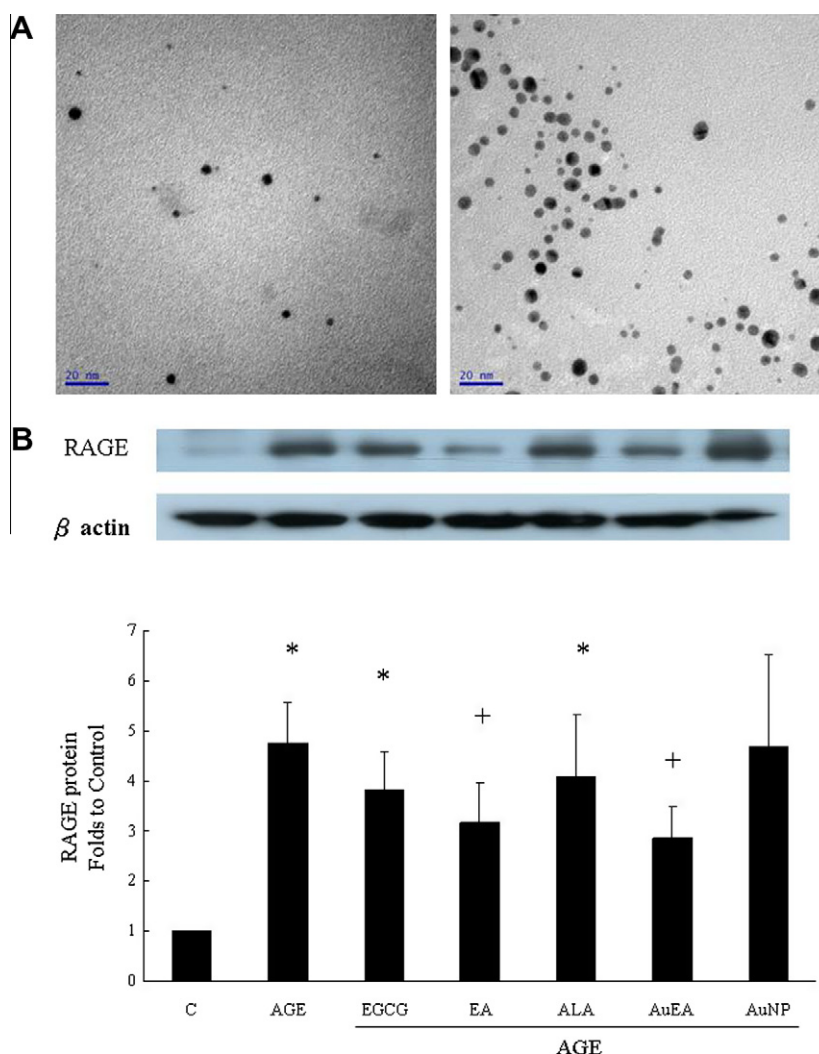


Fig. 1. EA and AuEA attenuates the AGE-induced RAGE expression in Hs68 cells. (A) The stability of the AuNP in the cell culture medium with serum was examined by transmission electron microscopy (TEM). The diameter of the utilized AuNPs were 3–5 nm. (B) Cultured Hs68 cells were treated with vehicle (C), EGCG, EGCG+ALA (EA), ALA, AuNPs+EGCG+ALA (AuEA) or AuNPs for 24 h. After EA and AuEA treatment, AGE-induced RAGE protein expression significantly decreased. ($n = 6$) * $p < 0.01$ when compared to control; * $p < 0.05$ when compared to AGE group.

scavenger of many reactive oxygen species (ROS) and a regenerator to other anti-oxidants such vitamin C and vitamin E (Lateef et al., 2005). ALA, existing in animal foods such as meat and liver (Kataoka, 1998), was discovered in 1951 as a coenzyme in the Krebs's cycle. Previous studies revealed its protective effects in aging, diabetes mellitus, vascular and neurodegenerative diseases all in which free radicals are involved (Hagen et al., 1999; Yilmaz et al., 2002). Nanotechnology is a branch of science and engineering dedicated to material with dimensions in the order of 100th of nm or less (Salata, 2004). Recently, nanotechnology has been applied in electronic storage systems (Kang et al., 1996) and biotechnology (Pankhurst et al., 2003). Gold nanoparticles (AuNPs) are a suspension of nanometer-sized particles of gold. Colloidal AuNPs have been proposed for diverse biomedical applications due to their unique surface, electronic, and optical properties (Daniel and Astruc, 2004; Eustis and el-Sayed, 2006). In recent years, AuNP served as vehicles for gene, biosensors, cancer cell imaging, photothermal therapy, and drug delivery (Anker et al., 2008; Huang et al., 2006; Pissuwan et al., 2006; Sokolov et al., 2003).

Topical drug application is always preferred in clinical cutaneous wound treatment due to less adverse effects on other organs. Topical application of ligands for several growth factors has been reported to enhance wound healing in animal models (Chigurupati

et al., 2007) and human subjects (Hong et al., 2006) successfully. In this paper, we evaluated the efficacy of topical use of AuNP, EGCG, ALA, or their mixture in mouse cutaneous diabetic wound healing. The possible underlying mechanisms were also studied.

2. Materials and methods

2.1. The preparation and characterization of AuNP

AuNP provided from Gold NanoTech Inc. (Taipei, Taiwan) were prepared by a proprietary molecular beam epitaxy process as follows. AuNP were produced by physical manufacturing and did not contain any surface modifiers or stabilizers. Briefly, gold bulk material was cut or ground into the target material. Then the Au target was vaporized to the atomic level by an electrically gasified method under vacuum. The vapor was condensed in the presence of inert gas and then piled up to form AuNP. The AuNP sizes can be effectively managed depending on the evaporation time and electric current used. The AuNP were collected in a cold trap and centrifuged to obtain the final product. The initial concentration of these AuNP was determined by an inductively coupled plasma mass spectrometer (ICP-MS, PE-SCIEX ELAN 6100 DRC, Waltham, MA, USA). The sizes of the various AuNP were observed by a JEOL

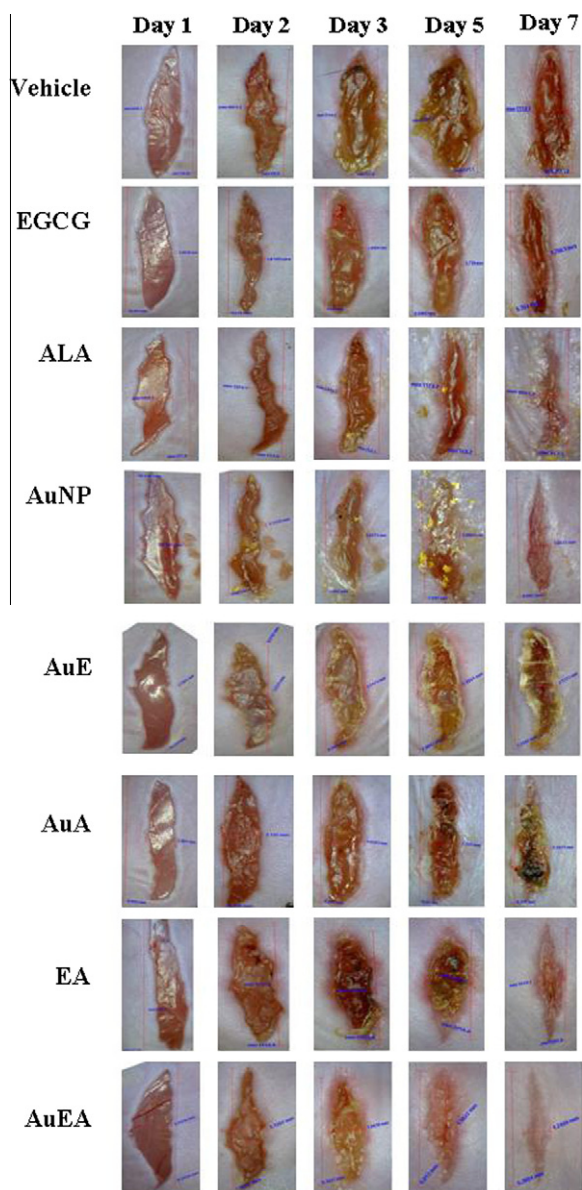


Fig. 2. Topical application of EGCG, ALA, AuNPs, AuE, AuA, EA and AuEA on the cutaneous wounds in diabetic mice. Two full-thickness wounds were induced in vehicle, EGCG, ALA, AuNPs, AuE, AuA, EA or AuEA-treated diabetic mice. Image representative diabetic mice were taken from injury first day to 7th day.

JEM-1200 transmission electron microscopy (TEM) (Tokyo, Japan) operated at 110 kV. The size distribution of the AuNP was computed by software based on more than 100 particles in the images. The UV–Vis absorbance of AuNP with various sizes was measured by UV–Vis spectroscopy (Hitachi U-2000, Tokyo, Japan). The negative surface charge of AuNP was determined by zeta potential (Zetasizer nano-zs, Malvern, Worcestershire, UK). The stability of the AuNP in the cell culture medium with serum was examined by transmission electron microscopy. The diameter of the utilized AuNP were 3–5 nm.

2.2. Cell culture

Human foreskin fibroblasts (Hs68) were cultured in DMEM (Cambrex Bio Science, MD, USA) containing 20% FBS, 100 µg/ml penicillin, and 100 µg/ml streptomycin (pH 7.6). Cultures were passaged on reaching 80% confluence, using 0.05% trypsin/EDTA

(GIBCO, Invitrogen, CA, USA) and the media was changed every 2 days. The experiments were performed at passage 3–5. Cells were incubated with either vehicle, 2.2 mM EGCG (E), 8 mM ALA (A), 2.2 mM EGCG+8 mM ALA (EA), 1 ppm AuNP or AuNP+EGCG+ALA (AuEA) for 24 h.

2.3. Diabetic full-thickness wounds and wound measurement

Male BALB/c mice were injected with STZ (250 mg/kg, intraperitoneal) in citrate buffer (pH = 4.5). Blood glucose levels were determined 7 days after STZ injection and only mice with blood glucose concentrations more than 16 mmol/l were used in the following study. All mice were maintained on a standard laboratory diet and water ad libitum. All mice were used experimentally when 8 weeks old and weight 20 g at the time of wounding. Mice were anesthetized using 2–2.5% vaporized inhaled isoflurane and the dorsal skin was cleansed with Betadine. Under sterile conditions, the dorsal area was totally depilated and single full-thickness excisional linear wound (1 cm) was created on the bilateral upper back of each diabetic mouse using a sharp scissors and a scalpel. The left wound served as control (vehicle treatment) and right wound treated with 1 mg/g EGCG (E), 30 mg/g ALA (A), 1 mg/g EGCG+30 mg/g ALA (EA), 0.07 mg/g AuNP or AuEA ointment applied directly to the wound site once daily in a blinded manner. All ointments composed with 2.5 ml of glycerol (Sigma Inc., MO, USA), 1 ml Creagel emulsifier (First Chemical, TPE, TW) and 45.5 dd H₂O. In order to determine different ointment-mediated healing efficiency, the residual wound size was measured from the unclosed wound area after 1, 2, 3, 5 and 7 days of topically using digital Dino calculation software (AM3013T Dino-Lite Premier, AnMo Electronics Corp., TW). Six mice in each group were euthanized on days 3, 5 and 7 post wounding and skin tissue samples from the wound site were excised in full depth and bisected from all of the mice for biochemical analyses or H&E histological staining. Total 135 mice were used in this study. The investigations conform to the Guide for Care and Use of Laboratory Animals published by the US National Institutes of Health and the Animal Care Committee of Institute for Frontier Medical Sciences, Fu-Jen University (A9658).

2.4. RNA isolation and reverse transcription (RT)

Total RNA was isolated from skin tissue or Hs68 cells using the single-step acid guanidinium thiocyanate/phenol/chloroform extraction method. For reverse transcription, 1 µg of RNA was incubated with 200 U of Script I reverse transcriptase (Ambo Life Inc., Taipei, TW) in a buffer containing a final concentration of 20 mmol/l Tris/HCl (pH 7.8), 100 mmol/l NaCl, 0.1 mmol/l EDTA, 1 mmol/l DTT, 50% glycerol, 2.5 µmol/l poly (dT)_{12–18} oligomer, and 0.5 mmol/l of each dNTP at a final volume of 20 µl. The reaction mixture was incubated at 45 °C for 1 h and then at 70 °C for 15 min to inactivate the enzyme.

2.5. Real-time PCR

The cDNA had a 10-fold dilution in nuclease-free water and was used for the Smart Quant Green Master Mix (Protech Technology Enterprise Co., Taipei, Taiwan): 2 µl of cDNA solution, 0.5 µmol/l primers, 5 mmol/l magnesium chloride, and 2 µl of Master SYBR-Green in nuclease-free water with a final volume of 20 µl. The primers used for PCR were: RAGE: forward, 5'AAGCCCTGGT GCCTAATGAG3', reverse, 5'CACCAATTGGACCTCTCCA3'; GAPDH: forward, 5'CGACCACTTTGT CAAGTCA3', reverse, 5'AGGGGTCTAC ATGGCAACTG3'. The initial denaturing phase was 5 min at 95 °C followed by an amplification phase as detailed below: denaturation at 95 °C for 10 s; annealing at 55 °C for 10 s; elongation at 72 °C for 15 s and detection at 79 °C for 45 cycles. Amplification,

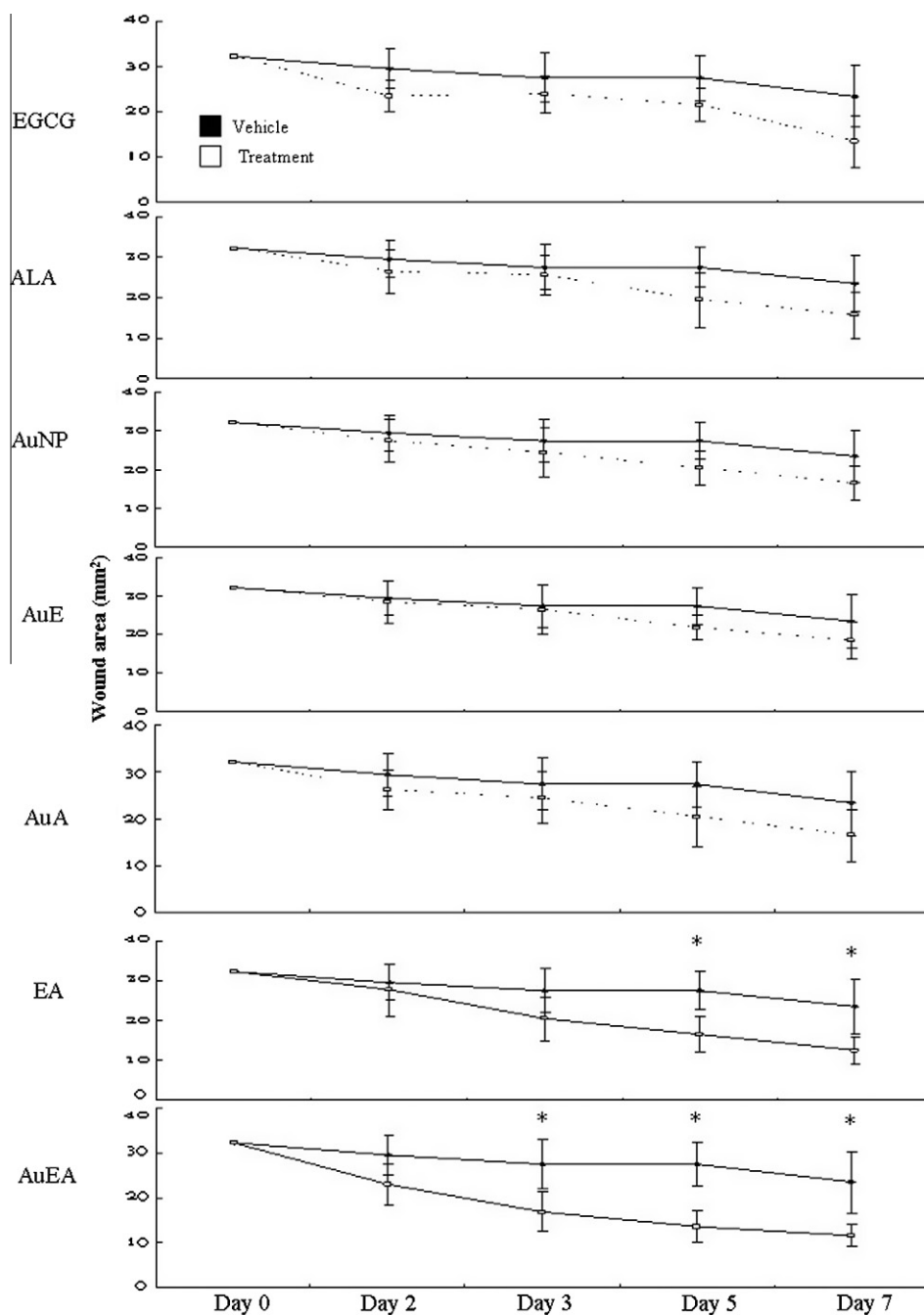


Fig. 3. EA and AuEA treatment decreased the wound area at the indicated time points. Topical application of EA and AuEA significantly accelerated the cutaneous wound healing in diabetic mice. Wound width and length significantly decreased from third day to 7th day in AuEA group. ($n = 6$) * $p < 0.01$ when compared to vehicle group.

fluorescence detection, and post-processing calculation were performed using the ABI Step1 apparatus. Individual PCR product was analyzed for DNA sequence to confirm the purity of the product.

2.6. Western blot analysis

Total protein samples were mixed with sample buffer, boiled for 10 min, separated by 10% SDS-PAGE under denaturing conditions, and electroblotted to nitrocellulose membranes (Amersham Pharmacia Biotech, CB, UK). The nitrocellulose membranes were blocked in blocking buffer, incubated with mouse anti-CD68 (AnaSpec Inc., CA, USA) anti-angiopoietin 1 (Ang-1), anti-angiopoietin 2 (Ang-2), and anti-vascular endothelial growth factor

(VEGF) (Santa Cruz Biotechnology Inc., CA, USA) antibody, washed, and incubated with horseradish peroxidase-conjugated secondary antibody. Signals were visualized by enhance chemiluminescent detection.

2.7. Statistical analysis

The data were expressed as mean \pm S.E.M. A Student's t -test was used for comparing parametric variables between the two groups, while ANOVA with repeat measurement design was used for time course changes. Statistical significance was evaluated by Turkey–Kramer multiple comparisons test (GraphPad Software Inc., San Diego, CA, USA). A p -value of less than 0.05 was considered statistically significant.

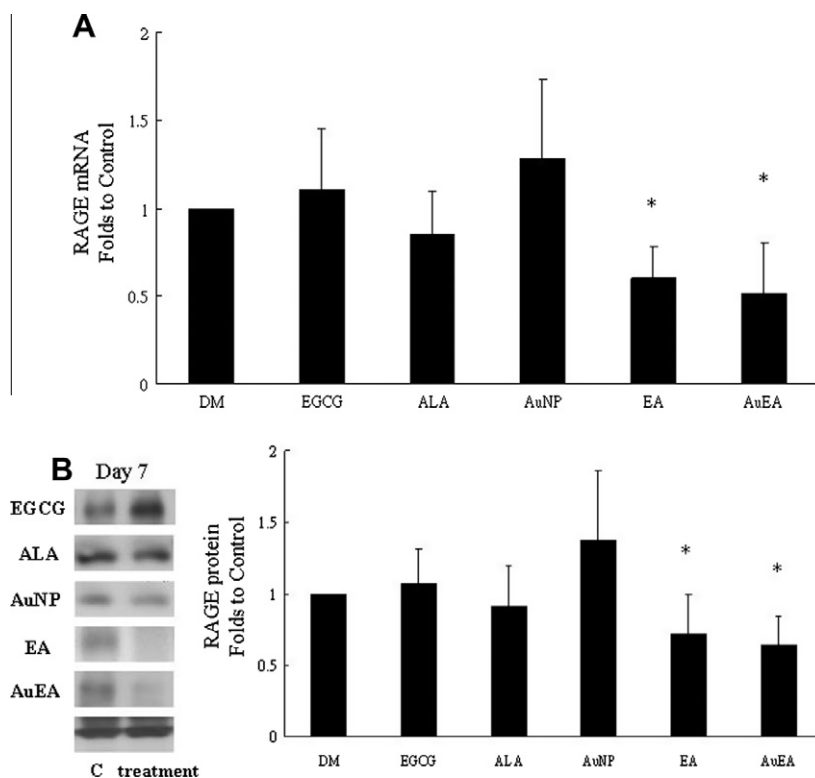


Fig. 4. RAGE expression in wound area after EA and AuEA topical treatment. RAGE expression significantly in the skin tissues from diabetic mice. Treated with EA and AuEA significantly attenuated RAGE mRNA (A) and protein (B) expression in diabetic wound area ($n = 6$). * $p < 0.01$ when compared to DM group.

3. Results

3.1. RAGE expression in AGE-treated Hs68 cells

We test the size and stability of AuNP in culture medium. The diameter of the utilized AuNP were 3–5 nm by TEM analysis (Fig. 1A). Incubation with AGEs significantly increased the RAGE protein expressions in Hs68 cells (Fig. 1B). Incubation with EGCG, ALA or AuNP alone 30 min prior to incubation with AGE did not significantly attenuate the RAGE protein expressions. However, the EA and AuEA group significantly decreased RAGE expression in AGE-treated Hs68 cells, respectively.

3.2. Effect of anti-oxidants and AuNP ointment on diabetic wound closure

For investigating wound healing process *in vivo*, two linear full-thickness wounds were created on the dorsum of vehicle (control), EGCG, ALA, AuNP, AuE (AuNP+EGCG), AuA (AuNP+ALA), EA or AuEA-treated diabetic mice (Fig. 2). The wound area were $34.25 \pm 1.1 \text{ mm}^2$ of Day 0 and $25.42 \pm 6.85 \text{ mm}^2$ of Day 7 in vehicle-treated diabetic mice. Topical application of EA and AuEA significantly increased the rate of diabetic wound healing than vehicle (Fig. 3). However, EGCG, ALA, AuNP, AuE and AuA treatment did not show significant between vehicles. Both wound widths and lengths in EA or AuEA-treated mice were significantly decreased from 5th to 7th day after injury when compared with vehicle. Furthermore, AuEA treatment significantly decreased the wound area after three days topical application. No adverse effects of topical treatment were noted on body weight, general health or behavior of the diabetic mice. At sacrifice, the level of blood sugar in experiment diabetic mice did not differ from the first day of cutaneous injury.

3.3. EA and AuEA mixtures decreased the skin tissue RAGE expression in diabetic mice

After topical application with EA or AuEA for a week, the skin tissues around the wound were collected for RAGE expression analysis. The RAGE mRNA levels increased in the skin tissue from diabetic mice. Both EA and AuEA treatment significantly attenuated the RAGE mRNA expression (Fig. 4A). Consistent with this finding, RAGE protein were significantly decreased by EA or AuEA treatment for a week in Western blot analysis (Fig. 4B). These data indicated that EA and AuEA topically used both attenuated the RAGE expression in the skin of diabetic mice during wound contraction.

3.4. VEGF increased by EA and AuEA topical application

By producing microvessels that provide nutrients and oxygen to growing dermal cells, angiogenesis may play a critical role in wound healing. To evaluate the EA or AuEA mechanism responsible for the diabetic wound healing, we analyzed angiogenesis gene expressions in the wound area. VEGF protein expression increased from day 5 to day 7 after injury in EA and AuEA group (Fig. 5A and Supplement 1A). The VEGF levels were higher in AuEA-treated group than EA-treated group on day 5 and day 7 after injury. However, Ang-1 protein only significantly expressed on day 3 compared to control site in EA-treated group (Fig. 5B). EGCG, ALA, EA or AuEA treatment did not significantly change the expressions of Ang-1 on day 7 (Supplement 1B). On the other hand, Ang-2 protein expression was different between EA and AuEA treatment. In Fig. 5C, EA treatment significantly increased the Ang-2 protein expression on day 5 and day 7 in diabetic wound area. However, AuEA treatment significantly increased Ang-2 expression on day 5 but decreased on

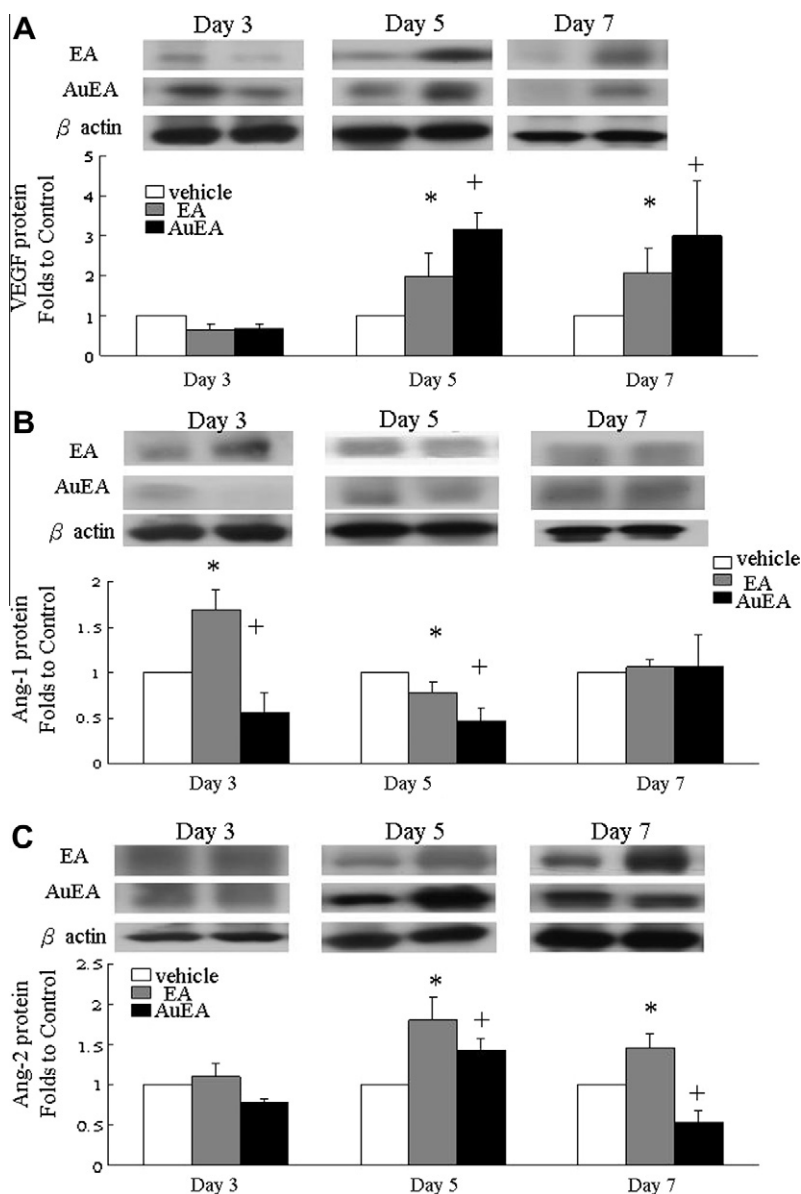


Fig. 5. Expression of angiogenesis related gene after EA and AuEA treatment in diabetic wound area. Western blot analysis for diabetic cutaneous wound area tissue at different time point after EA and AuEA treatment. (A) VEGF protein expression significantly increased from day 5 to day 7 post-injury after EA and AuEA treatment. ($n = 6$). (B) Angiopoietin-1 significantly decreased on day 3 and day 5 in AuEA group. ($n = 6$). (C) Angiopoietin-2 protein significantly increased on day 5 in EA or AuEA treated group. The expression of Ang-2 significantly decreased on day 7 in AuEA but not EA group. ($n = 6$) * $p < 0.05$ EA group compared to vehicle group. * $p < 0.05$ AuEA group compared to vehicle group.

day 7. On day 7, AuNP treatment also significantly decreased the Ang-2 expressions (Supplement 1C).

3.5. EA and AuEA decreased the inflammation in diabetic wound area

To examine the possible roles of EA or AuEA treatment in the wound inflammation, CD68 expression were used for marker of monocyte infiltration (Fig. 6A). Both EA and AuEA group significantly decreased the CD68 expression from day 3 to day 7 after cutaneous injury in diabetic mice. The effects of EA treatment were similar to those of AuEA treatment on attenuated CD68 expression. The EGCG and AuNP treatments also show the ability to decrease CD68 expression on Day 7 (Supplement 2). In histological sections of skin samples from AuEA group, neutrophil infiltration and inflammation was limited to the site of wounding on day 7 (Fig. 6B).

4. Discussion

Type 2 diabetes affects almost 4% of the world's population and diabetic patients are at high risk for developing foot ulcers. The rates of amputation range from 43.9 per 100,000 per year among diabetic patients in America (Gravely et al., 2011). The formation of AGE has been recognized as an important pathophysiological mechanism in the development of diabetic ulcers; the binding of circulatory AGE to RAGE on different cell types leads to impaired function of growth factors. Glycation is an important pathway in the pathogenesis of microvascular and macrovascular complications of diabetic foot ulcers and the oxidative stress-related AGE/RAGE interaction may play a key role (Huijberts et al., 2008). We therefore investigated the effects of the anti-oxidant mixture EGCG and ALA with AuNP on diabetic wound healing. The angiogenesis pathway was also evaluated because it plays an important role in the activation of wound healing.

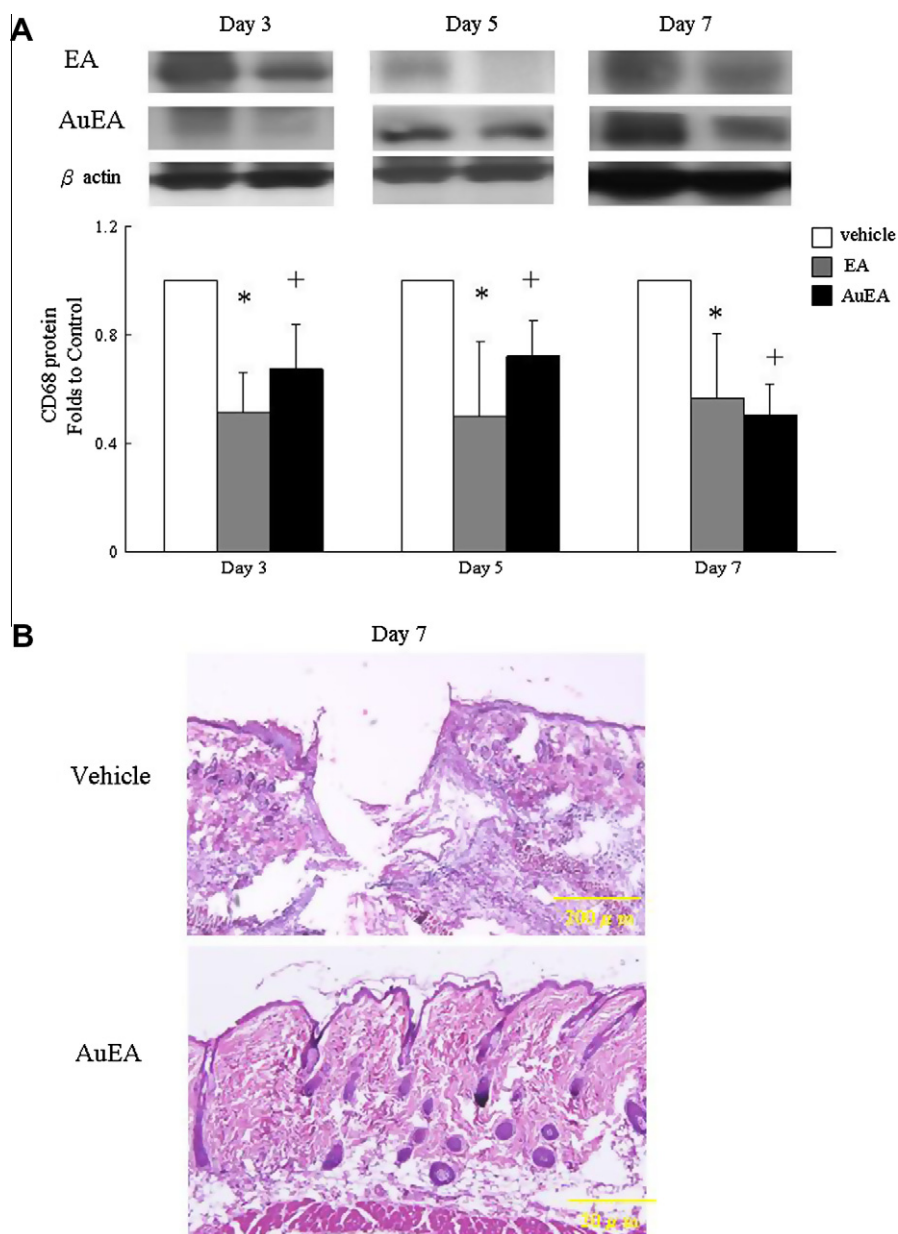


Fig. 6. Anti-inflammatory effect in wound tissue from diabetic mice treated with EA or AuEA. (A) CD68 protein significantly decreased after EA or AuEA treated from day 3 to day 7 post-injury. ($n = 6$). (B) Topically treated with AuEA did not lead to abnormal host response. H&E-stained skin sections were examined microscopically from AuEA-treated mice 7 days after wounding. No significantly increase in neutrophil infiltration was noted in tissues adjacent to the wound site in AuEA group after wounding. Original magnification 40 \times (vehicle) and 400 \times (AuEA). * $p < 0.01$ EA group when compared to vehicle group. ⁺ $p < 0.05$ AuEA group compared to vehicle group.

The compound 3-deoxyglucosone is the precursor for AGE and is greatly upregulated in diabetic cutaneous wounds and has been shown to make the dermal fibroblasts dysfunction (Loughlin and Artlett, 2010). In type 2 diabetic skin tissues the expression of both AGE and RAGE was increased when compared with normal skin tissues. Further study in human dermal fibroblasts demonstrated that cell arrest and apoptosis was increased (Niu et al., 2008). The blockage of RAGE by intraperitoneal soluble RAGE significantly suppressed the tumor necrosis factor α and interleukin-6 while enhancing cutaneous wound closure in *db/db* mice (Goova et al., 2001). Topical treatment with soluble RAGE to bind the AGE significantly improved the process of wound healing in *db/db* mice in the full-thickness excisional wound model (Wear-Maggitti et al., 2004). This research suggests that interaction of the AGE with RAGE can trigger an inflammatory response and compromised collagen production. This leads to impaired diabetic wound healing.

Control of the inappropriate inflammation, oxidative stress and vascular injury may be a key in managing diabetic wound ulcers. In this study the combination of EGCG, ALA and AuNP in specific concentrations significantly decreased expression of the RAGE protein within cultured fibroblasts and in diabetic full-thickness excisional cutaneous wound areas. The study from Matsui also demonstrated that vildagliptin was able to decrease the vascular injury in diabetic rats by attenuating the effects of the AGE-RAGE-oxidative stress axis (Matsui et al., 2011).

High glucose impaired cell migration is due to an increase in oxidative stress that causes deficient adhesion of skin fibroblasts; sustained hyperglycemia in diabetic wound areas leads to an increased production of vascular superoxide (Lamers et al., 2011). The new therapeutic strategies to decrease the production of oxidative stress provide new hope in improving impaired diabetic wound healing. More recently a cell therapy study demonstrated

that diabetic endothelial progenitor cells accelerated wound healing in type 2 diabetic mice after manganese superoxide dismutase gene transfer (Marrotte et al., 2010). The intraperitoneal anti-oxidant 150 mg/kg N-acetyl cysteine for 5 days significantly promoted cutaneous wound healing in diabetic mice (Aktunc et al., 2010); therefore, we topically applied the anti-oxidant EGCG combined with ALA and AuNP on the cutaneous diabetic wound. In our previous study, EGCG decreased RAGE mRNA and protein expression in AGE-treated human mesangial cells (Liang et al., 2010). 5 μ M EGCG also attenuated AGE-induced RAGE in neuronal cells (Lee and Lee, 2007). Another anti-oxidant, ALA, also effectively protected the β cells and attenuated the damage of diabetic nephropathy in mice (Yi et al., 2011). RAGE-induced ROS, caspase-3 activation and nuclear DNA degradation in diabetic ganglia neurons was prevented by ALA treatment (Vincent et al., 2007). A recent study demonstrated that co-administration with AuNP enables percutaneous delivery of this protein drug (Pankhurst et al., 2003). These studies suggest that anti-oxidants agents for diabetic ulcer treatment may be a potential therapeutic application. Furthermore, AuNP may increase the specific mixture absorption through the skin by opening the stratum corneum transiently as AuEA significantly increased cutaneous wound closure in diabetic patients. These findings suggest that a topical application of AuEA was more effective than using anti-oxidants or AuNP alone.

Nanotechnology has recently attracted attention in diabetic wound research. Koria applied a keratinocyte growth factor chimeric nanoparticle to full thickness wounds in diabetic mice (Koria et al., 2011). The utilization of recombinant human epidermal growth factor nanoparticle promoted full-thickness wound repair in diabetic rats (Chu et al., 2010). Liu topically applied silver nanoparticles that significantly improved the wound healing processes (Liu et al., 2010). Previous studies have shown that AuNP could be applied therapeutically for intravascular and/or percutaneous drug/gene delivery (Pankhurst et al., 2003). We attempted to investigate the effect of AuNP combined with anti-oxidants in the diabetic wound healing processes. The anti-inflammation effect did not significantly differ between EA and AuEA group. In this study, we suggest that AuNP may increase the specific mixture absorption through the skin of a diabetic cutaneous wound not to augment the anti-inflammatory effect.

Angiogenesis is a critical step in cutaneous wound healing. The angiopoietin family is a critical regulator of blood vessel development in wound healing. Impaired wound healing in diabetic rats was correlated with an abnormal overexpression of Ang-2 and a down-regulation of VEGF (Qiao et al., 2011). In a *db/db* mice study impaired diabetic healing conditions were associated with the dysregulation of Ang-1, Ang-2 and VEGF expression (Kampfer et al., 2001). Therefore, we investigated the angiogenic effect of AuEA in diabetic cutaneous wound healing. In our study, AuEA significantly increased the expression of VEGF but not of Ang-2 in the diabetic wound area. Both EA and AuEA groups regulated Ang-1 into constant expression at day 7. Furthermore, Ang-2 significantly decreased on day 7 in AuEA treatment. Ang2 may counteract Ang1-mediated blood vessel stability, thus maintaining the endothelium in a more plastic state and promoting the response of endothelial cells to angiogenesis growth factor (Holash et al., 1999). After AuEA treated, the expressions of Ang2 and VEGF increased during the same period on day 5 may provide the drive for sprouting angiogenesis. The later decrease of Ang-2 and recovery of Ang-1 expression on day 7 may augment the angiogenic signal pathways interaction with VEGF to modulate vessel remodeling after cutaneous wound. Ang-2 but not Ang-1 increase in the early phase may suggest that AuEA had greater influence on Ang-2-induced angiogenesis. The combination of EGCG, ALA and AuNP in specific concentrations may delicately regulate the angiogenic effects in the molecular level on diabetic cutaneous wound. This data

suggests that a topical application of AuEA turns the new angiogenic vessel into a mature nonleakage vessel during the diabetic wound healing processes.

In summary, this study has provided insight into the molecular action of anti-oxidants used in a mixture with AuNP in diabetic cutaneous wounds. We have demonstrated that AuEA significantly accelerated diabetic wound healing through anti-inflammation and angiogenesis modulation. AuNP may serve as an adjuvant to increase the skin absorption and the functional ability of anti-oxidants. Diabetic foot ulcers remain a complex problem in clinical settings and this study strongly supports the beneficial effects of anti-oxidants and nanoparticles on diabetic patients with cutaneous wound and clearly provides a potential therapeutic application for the topical use of AuEA in cutaneous wound therapy.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at <http://dx.doi.org/10.1016/j.ejps.2012.08.018>.

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